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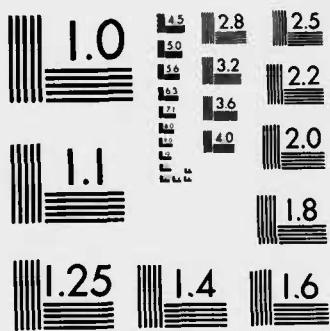
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STUDIES ON THE ENZYMATIC HYDROLYSIS OF ORGANOPHOSPHATE POISONS
IN PIGS

Part 1. pH and Ion Effects in Sera from Pigs, Rats, and Humans

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and
JUNE JAEGER, BS

CHEMICAL DEFENSE GROUP
DIVISION OF CUTANEOUS HAZARDS

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**Studies on the Enzymatic Hydrolysis of Organophosphate Poisons in Pigs. Part I. pH and ion effects
in sera from pigs, rats, and humans-Schmid and Jaeger**

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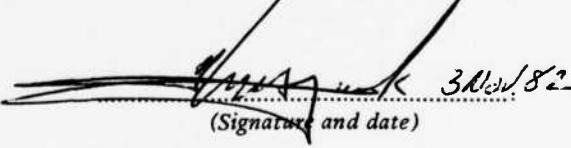
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ABSTRACT

Hydrolysis of the organophosphate paraoxon was studied in Yorkshire pig, rat, and human sera. Enzymatic hydrolysis was significantly affected by diethylmalonate and TRIS buffer in the reaction mixture. Kinetic measurements suggest that more than one enzyme hydrolyzes the organophosphate. Depending on pH and substrate concentration, inhibition of the enzyme by paraoxon is or is not observed. Enzymatic activity in human serum increases with a rise in pH (6.7 to 10.2) whereas in pig serum activity decreases. In glycine buffer enzymatic activity in rat serum is approximately equal to that of pig serum.

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PREFACE

This is the first in a series of reports that will be concerned with the biochemical response of Yorkshire pigs to organophosphate poisoning. For comparison, data are also given for rat and human sera. Subsequent reports will be concerned with the biochemical responses of skin to organophosphate poisoning. These basic data are needed to develop new decontamination or prevention strategies against chemical warfare agents.

We wish to thank Nancy E. Sarfi for secretarial assistance in the preparation of the transcript, and Lottie B. Applewhite, M.S. for many editorial suggestions.

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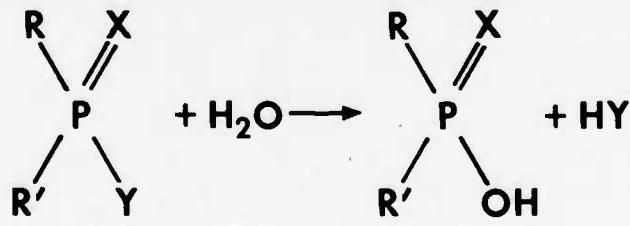
STUDIES ON THE ENZYMATIC HYDROLYSIS OF ORGANOPHOSPHATE POISONS IN PIGS.
Part I. pH and Ion Effects in Sera from Pigs, Rats, and Humans—Schmid and Jaeger

To understand the toxic effects of organophosphates (OP) on skin and to develop effective skin decontamination systems against chemical warfare agents, knowledge of the metabolism of OP in skin is necessary. Progress in skin decontamination technology depends on the use of realistic and relevant animal models. Pig skin in many ways has a morphology similar to human skin and therefore is potentially an important animal model to test skin decontamination systems. Few data have been reported on the effect of OP on pig tissues.

In initial studies in this laboratory, we used a variety of strategies to homogenize and measure phosphoryl phosphatase in pig skin. We encountered a number of difficulties which suggested the necessity for a study of phosphoryl phosphatase in pig serum.

Organophosphates inhibit many enzymes in addition to acetylcholinesterase, and a number of enzymes hydrolyze OP (1,2). Enzymes have been detected that hydrolyze chemical warfare agents (G-agents and V-agents) as well as chemicals, such as diisopropylfluorophosphate (DFP). Numerous names have been given to these enzymes such as phosphotriesterase, phosphoryl phosphatase, paraoxonase, DFP-ase, tabunase or somanase.

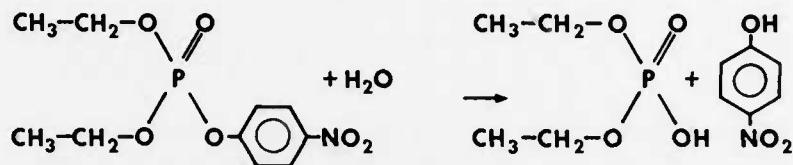
A common feature of all these enzymes is that they are able to hydrolyze OP according to the general reaction.



where, X is oxygen or sulfur in certain insecticides, Y is a leaving group such as fluorine, cyanine or paranitrophenol, and R and R' are alkyl or alkoxy groups.

Since at this time we are unable to study chemical warfare agents

such as tabun we are using as a realistic biologically active simulant - the OP paraoxon, which is a cholinesterase inhibitor. Hydrolysis of paraoxon yields 1 mole of p-nitrophenol according to the equation



and the rate of hydrolysis can be measured spectrophotometrically by the appearance of p-nitrophenol.

The influence of pH on the hydrolysis of paraoxon in serum of a variety of mammals has been studied by a number of investigators (3-6). The enzyme in rabbit serum shows maximal activity at pH 7.4 (3). In contrast the isolated and purified enzyme from sheep serum has its highest activity at pH 5.0 and appears to be stable between pH 4 and 10 (4). The enzyme in human serum has a maximum activity at pH 11 (5). These reports (3-7) suggest that several serum enzymes exist which hydrolyze OP in sera. It is also possible that large differences between mammals exist. The first possibility is suggested by the data of Erdős and Boggs (6) who showed that serum fractions prepared by the method of Cohn et al (8) have different enzymatic activity. Various fractions are also affected differently by inhibitors and activators.

The variability in data regarding the properties of phosphoryl phosphatase and the lack of information for pig sera suggested a systematic study of this enzyme. In this report we present results on the effect of pH on the hydrolysis of paraoxon in pig serum and compare these results with those obtained with rat and human serum.

MATERIAL AND METHODS

Pig Serum. Yorkshire pigs weighing about 25 to 35 lb (11.34 to 15.91 kg) were sacrificed by intravenous injection of 3 ml of Sleepaway^(R) (Fort Dodge Laboratory, Iowa). Blood was collected by heart puncture. Pig blood was allowed to clot at 25°C for 50 min. Serum was collected and stored in the same manner as human blood serum. Experiments were done with serum from individual pigs.

Rat Serum. Sprague-Dawley rats weighing 300 g were anesthetized lightly with ether and blood was removed from the jugular vein. Tubes containing the blood was allowed to clot at 25°C for 50 minutes. Serum was collected and stored in the same manner as human blood serum.

Human Serum. Blood was obtained from 5 healthy, Caucasian volunteers. Their average age was 50 years. Venous blood was collected in 13 ml red top Vacutainer^(R) tubes and allowed to clot at 25 C for 50 min. Serum was collected after centrifugation at 2000 rpm for 20 min in an International Equipment Company Centrifuge (Needham Heights, Maine). Sera were clear and showed little evidence of hemolysis. Sera were stored at -20 C.

Chemicals were of analytical grade and were obtained from Sigma Chemical Company (St. Louis, Missouri) with the exception of paraoxon and p-nitrophenol which were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin), CHES (cyclohexylaminoethanesulfonic acid) A grade was purchased from Calbiochem Behring Corporation (La Jolla, California).

Determination of Absorptivity Constant for P-nitrophenol. P-nitrophenol was dissolved in 100% acetonitrile and aliquots were added to 3 ml of 0.1 M TRIS buffer (pH 6.6, 7.0, 7.3, 7.6, 8.0, and 8.7), 0.1 M CHES buffer (pH 8.7 and 9.2) and to 0.1 M glycine buffer (pH 8.7, 9.2, 9.7, 10.2, and 10.7). Spectra were measured between 500 and 350 nm at 32 C with an Acta III Spectrophotometer (Beckman Instruments, Inc., Fullerton, California).

Determination of Enzymatic Activity. Hydrolysis of paraoxon was followed by measuring the liberation of p-nitrophenol at 400 nm. A stock solution of paraoxon 0.37 M was prepared by dissolving the pure liquid in acetonitrile. Acetonitrile did not influence the enzymatic reaction (data not shown). Rates were measured in 0.1 M buffer and the incubation temperature was kept at 32 C. Rates were calculated from recordings of absorbance as a function of time. Rates were corrected for nonenzymatic hydrolysis of paraoxon and expressed in moles per liter per minute.

Determination of Protein. The concentration of protein was determined by the method of Lowry et al (9). Serum was diluted 1.20 and bovine albumin fraction V (Sigma Chemical Company, St. Louis Missouri) was used as a standard. Duplicate protein determinations were run on each sample.

RESULTS AND DISCUSSION

pH Dependence of Absorptivity of p-nitrophenol. At 25 C the dissociation constant for p-nitrophenol is reported as 7.15 (10). Since the absorptivity of the p-nitrophenolate ion is much higher than that of p-nitrophenol, it is important to know the absorptivity constant as a function of pH. Figure 1 shows the relationship of absorptivity to pH of p-nitrophenol solutions. The absorptivity constant increased from about $\epsilon = 4000$ at pH 6.6 to a value of 19000 above a pH of 8.7. The mean value between pH 8.7 and 10.7 was 19000 with a standard deviation of 1500. Below pH 8.7 a TRIS buffer was used

and above pH 8.7 a glycine buffer was used. At pH 8.7 and 9.2 the absorptivity was not affected by the buffer ions (CHES, TRIS, glycine) at 0.1 M (data not shown).

Spectral Changes in Absorbance During Enzymatic Hydrolysis of Paraoxon. We wanted to see if there were changes in the absorption spectrum of p-nitrophenol in the presence of serum. In Figure 2 ultraviolet spectra for the components of the enzymatic assay are shown. The curves indicate that hydrolysis of paraoxon to p-nitrophenol can be followed by measuring changes in absorbance at 400 nm. Figure 2 shows spectra at pH 7.6. Similar spectra were obtained at pH 6.6, however, absorptivity was considerably lower (Fig 1) than at pH 8.7. Above pH 9.5 the competing non-enzymatic hydrolysis of paraoxon became significant and at pH 11.0 the net rate became uninterpretable.

Influence of Pig Serum Concentration on Reaction Rates. The influence of the concentration of pig serum on the rate of hydrolysis of paraoxon at a constant concentration of 1.2×10^{-3} M was tested at pH 10.7. Figure 3 demonstrates the rate of hydrolysis was proportional to the amount of serum used. The slope for the line representing initial rate is 7.0×10^{-9} moles per liter per minute per microliter serum. For longer time intervals ($t_{20} - t_5$ min), the slope decreases to 3.7×10^{-9} . The initial rates were significantly higher than rates calculated with longer time intervals. If there is more than one phosphorylphosphatase in pig serum such kinetic behaviors could be expected. However, since the overall objective was to study the action of OP on pig skin, purification and isolation of the enzyme(s) was not initiated.

For comparison, limited tests on serum from 5 human volunteers were done. Two volumes of serum of 60 and 180 μ l were used and data corrected for non-enzymatic hydrolysis at pH of 10.6. In Table 1 initial rates (moles per liter per minute per microliter serum) indicate rates of hydrolysis of paraoxon in human sera are also proportional to the amount of enzyme. However, in order to measure rates accurately the paraoxon concentration had to be reduced from 1.23×10^{-3} to 3.7×10^{-4} M. The data from Figure 3 and Table 1 are thus not directly comparable but suggest a significantly higher rate of hydrolysis for human serum. There was a significant range of activity in the 5 sera. This is compatible with the findings and explanation of Krisch (5) who found a bimodal distribution of the enzymatic activity in healthy volunteers.

Influence of Buffer Ions on Enzymatic Hydrolysis of Paraoxon. In the hydrolysis of paraoxon, acid is produced and thus a constant pH and buffer capacity are important to maintain a constant pH. However, ion specific effects of the buffer may either inhibit or activate the enzymes that hydrolyze paraoxon. The buffer may also not be compatible with metal activators such as calcium ions, leading to the formation of

precipitates. For this reason we compared rates of hydrolysis of paraoxon in 0.1 M phosphate, 0.1 M diethylmalonic acid and 0.1 and 0.01 M in TRIS buffers all at pH 7.4 (Table 2). Measurements were made with 1.48×10^{-3} M paraoxon, a concentration well below its limited solubility. Column 2 shows a wide range of activity from 0.8 to 6.4×10^{-7} moles per liter per minute per milligram protein. For comparative purposes relative rates are given in Columns 3 to 6. The data indicated that rates are considerably lower in 0.1 M diethylmalonic acid and 0.1 M TRIS buffer than in phosphate buffer. Comparison of columns 5 and 6 suggests that use of the lower concentration of TRIS results in significantly higher activity. Choice of buffer, however, is important if activation by metals such as calcium is studied. Since activity in TRIS is higher than in diethylmalonic acid buffer at pH 7.4 and since TRIS buffer is compatible with metal ions, it was chosen for most of the determinations at lower pH.

Influence of Paraoxon Concentration on Hydrolysis in Pig Serum at pH 7.4. Paraoxon has a limited solubility of 8.72×10^{-3} M in pure water (11). However, solubility in salt or protein solutions may be quite different. Such data were not available but our observations suggest a limited solubility in 0.1 M TRIS buffer of about 5×10^{-2} M whereas in protein solutions the value was 10^{-2} M. This suggests that in 0.1 M buffers a reduction in solubility occurs. In contrast, considerable binding of paraoxon to serum protein occurs in dilute protein solutions. Depending on the order of addition of paraoxon to the assay solutions paraoxon may or may not stay in solution.

The kinetics of hydrolysis of paraoxon in pig serum-TRIS buffers at pH 7.6 are complicated. At the time of mixing, a significant amount of p-nitrophenol is liberated. This liberation occurred in less than 0.2 minute and was concentration dependent (Fig 4a) between 0 and 10^{-2} M paraoxon. This suggests an initial rate of about 1.3×10^{-7} moles per liter per minutes. Between 0.2 minutes and 10 minutes the rate decreased and over the period of 10 to 180 minutes the rate of release of p-nitrophenol followed zero order kinetics. In Figure 4b data are plotted in the double reciprocal manner $1/v$ versus $1/s$. For this Lineweaver-Burk plot, V_{max} is 3.7×10^{-7} moles liter per minutes and K_m is 0.028 M. At a 10^{-2} M paraoxon concentration, which is close to the limited solubility of paraoxon in serum solutions, the initial and the slower rate constants differed by a factor of approximately 10.

Concentration Dependence of the Hydrolysis of Paraoxon in Glycine-Buffer pH 10.5. Below the limited solubility of paraoxon, i.e. over a concentration range of 0 to 8×10^{-3} M paraoxon, the rate of hydrolysis in pig serum showed a maximum value at a concentration of about 3×10^{-3} M. Above that concentration the rate decreased markedly (Fig 5). However, it appears that this decrease was not due to limited solubility effects. At this time there are no additional data that explain the maximal activity at 3×10^{-3} M and the reduced activity at

a higher paraoxon concentration. However, the reduced activity may be due to product inhibition from the liberated p-nitrophenol. Such a substrate inhibition was reported for the hydrolysis of acetylcholine by choline esterase (12).

A more detailed analysis over the limited concentration range of 0 to 3×10^{-3} M paraoxon was made with another serum (Fig 6). Since the initial rate changes rapidly, rates were calculated for three time intervals i.e. initial time interval, the interval between 5.3 and 0.3 min and the interval between 20 min and 5 min. Figure 6 indicates that the kinetics are complex. At low substrate concentrations up to about 1.0×10^{-3} M paraoxon, the initial rate and the rate given by the time interval $t_{5.3} - t_{0.3}$ were essentially equal whereas the rates for longer time intervals $t_{20} - t_5$ were clearly much lower. The V_{max} value for the three time intervals was different. If the Dixon procedure (13) is used to determine K_m and V_{max} from experimental observations, the values shown in Table 3 can be obtained from the data. V_{max} decreased over the period of the reaction by about 30%, whereas, smaller changes in K_m were observed. A rigid comparison of data is not possible between the K_m of 3.2×10^{-4} M for human blood reported by Krisch (5) and the values reported here.

Comparison of Pig, Rat and Human Sera. In Table 4, rates of hydrolysis are given for TRIS and glycine buffers. It appears from these data that the hydrolytic activity for paraoxon in human sera was significantly higher than in the two animal species. This appears to be true for measurements made at pH of 7.5 and 10.5. The error for assays at pH 7.5 was much smaller than at pH 10.5 at which a small difference in pH between the non enzymatic and enzymatic rate affected the net rate considerably. In contrast, at pH 7.5 the nonenzymatic hydrolysis was small. Serum values for the three volunteers differed considerably (Tables 1 and 4). It also appeared that the pH may affect the enzymes of the sera of volunteers differently. Aldridge (3) gives data for hydrolysis of paraoxon in Sorensens phosphate buffer pH 7.4. Although absolute values cannot be compared with the data reported (3), the relative values for rabbit, human, and rat were 4.05, 0.58 and 1.0 respectively. These data suggest that rat serum and human serum showed comparable activity and that rabbit serum has a much higher activity. Zech and Zuercher (7), unlike Aldridge, (3) used calcium ions in their measurements. Zech and Zuercher's data (7) suggest that V_{max} for pooled human serum and rat serum at pH 7.4 differs by a factor of 5. However, Erdős and Boggs (6) showed that calcium ions increased the hydrolysis of paraoxon. Thus our data are more in line with those of Zech and Zuercher (7); these investigators however, did not report data on pig serum.

pH Dependencies of Enzymatic Activity Between pH 6.6 and 11.5. Enzymatic activity over an extended pH range is not available. Although this report does not deal with skin nor decontamination of skin, it is important to know how pH affects enzyme activity since most

decontamination systems work at high pH. Furthermore data from Aldridge (3), Main (4), and Krisch (6) indicate different maximal activity for various sera. Our data (Table 4) suggest significant differences for pig and human sera. For this reason we studied the influence of pH in more detail.

Since the absorptivity constant ϵ for p-nitrophenol formed during hydrolysis from paraoxon (Fig 1) changes absorbance values were corrected and rate data expressed in moles per liter per minute per milligram protein (Fig 7). Rates of hydrolysis were determined at a fixed concentration of serum protein and paraoxon concentration of 2.6×10^{-4} M paraoxon per milligram human serum protein and 3.6×10^{-4} M paraoxon per milligram pig serum protein.

All data were obtained with single samples of pig and human sera. However, measurements were randomized so that a possible loss in enzymatic activity would not bias the data.

The data indicate a complicated relationship between pH and the rate of hydrolysis for human serum. The results suggest 3 maxima at pH 7.4, 8.7, and 10.0. Above pH 11.0 the rate of the nonenzymatic hydrolysis increased rapidly. Since rates for sera were corrected for nonenzymatic activity the rates become subject to considerable error. Between pH 10.4 and pH 11.4 the rate decreased by a factor of about 10. This suggests that a phenolic hydroxyl group on the enzyme(s) may be assisting in the hydrolysis of paraoxon. Maximal activity at pH 8.5 may be related to involvement of a protonated sulphydryl group. The pig enzyme was considerably less active than the human enzyme(s) and the rate declined from pH 6.6 to about pH 10.0 with questionable increase between pH 10 and pH 11.

CONCLUSIONS

- Rates of enzymatic hydrolysis of paraoxon are affected by different buffers in the reaction medium.
- Above pH 7.0 the direct spectrophotometric enzymatic method becomes quite sensitive.
- Between pH 6.0 and pH 8.5 the absorptivity coefficient for p-nitrophenol increases and reaches a constant value above pH 8.5.
- At pH 7.6 at least two enzymatic reactions, whose rates differ by a factor of 10, are observed in pig serum.
- Enzyme inhibition above a concentration of paraoxon of 3×10^{-3} M is observed at pH 10.5. Such enzyme inhibition is not observed at pH 7.6.
- The pH profile for paraoxon hydrolysis in human serum differs from that in pig serum. Rates of hydrolysis for pig and rat serum are significantly lower than for human serum.

RECOMMENDATIONS

Recommendations will be made in papers appearing later in this series.

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FIGURES

- Figure 1. Effect of pH on the absorptivity constant of p-nitrophenol in buffers.
- Figure 2. Ultraviolet-visible spectra of components in the reaction of paraoxon with pig serum in TRIS buffer pH 7.6.
- Figure 3. Dependence of rate of hydrolysis on pig serum concentration in glycine buffer pH 10.7.
- Figure 4. Hydrolysis of paraoxon with pig serum (3.4 mg/ml) in TRIS buffer pH 7.6.
- Initial liberation of p-nitrophenol (M) for paraoxon concentrations (M).
 - Lineweaver - Burk plot.
- Figure 5. Influence of paraoxon concentration (M) on rate of hydrolysis (moles per liter per minute) in pig serum and glycine buffer (pH 10.5).
- Figure 6. Influence of paraoxon concentration (M) on the rate of hydrolysis (moles per liter per minute) in pig serum.
- Figure 7. PH profile for the hydrolysis of pig serum (X) and human serum (O).

APPENDIX A

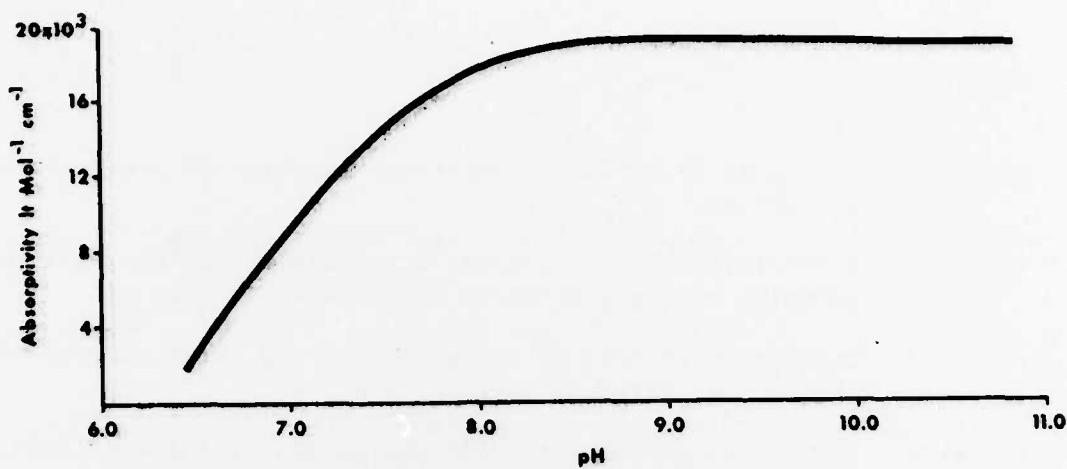


Figure 1. Effect of pH on the absorptivity constant of p-nitrophenol in buffers. Absorptivity in liters per mole per centimeter.

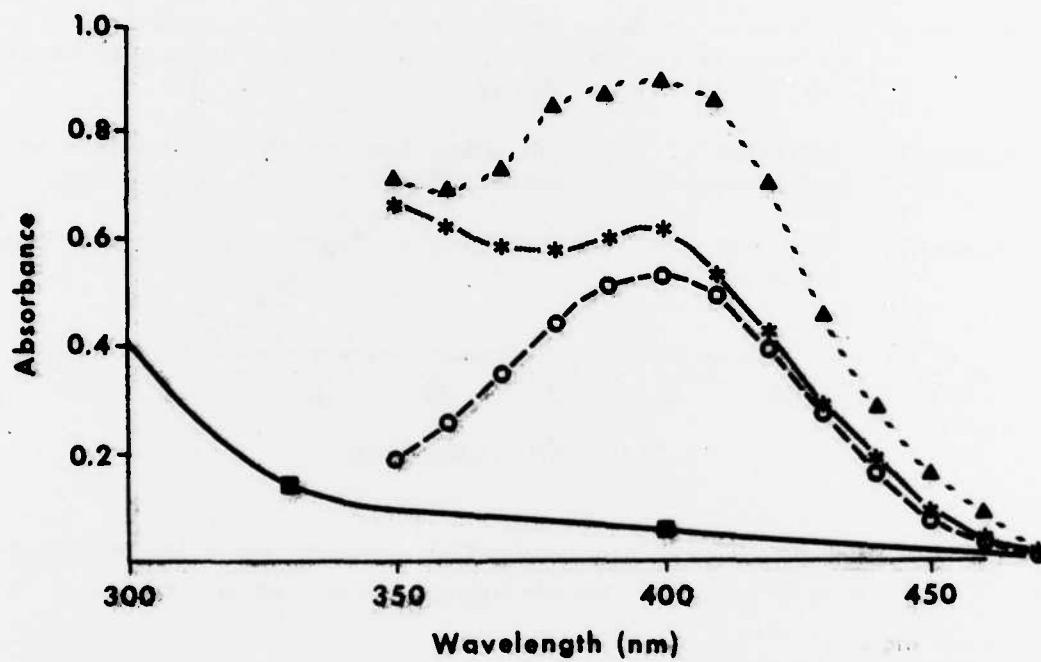


Figure 2. Ultraviolet-visible spectra of components in the reaction of paraoxon with pig serum in TRIS buffer pH 7.6.

- $7 \times 10^{-5} M$ paraoxon
- $3.5 \times 10^{-5} M$ p-nitrophenol
- ◆ Spectrum 1 min after addition of $1.48 \times 10^{-3} M$ paraoxon to 3.4 mg/ml serum protein
- ▲ Spectrum 55 min after addition of $1.48 \times 10^{-3} M$ paraoxon to 3.4 mg/ml protein

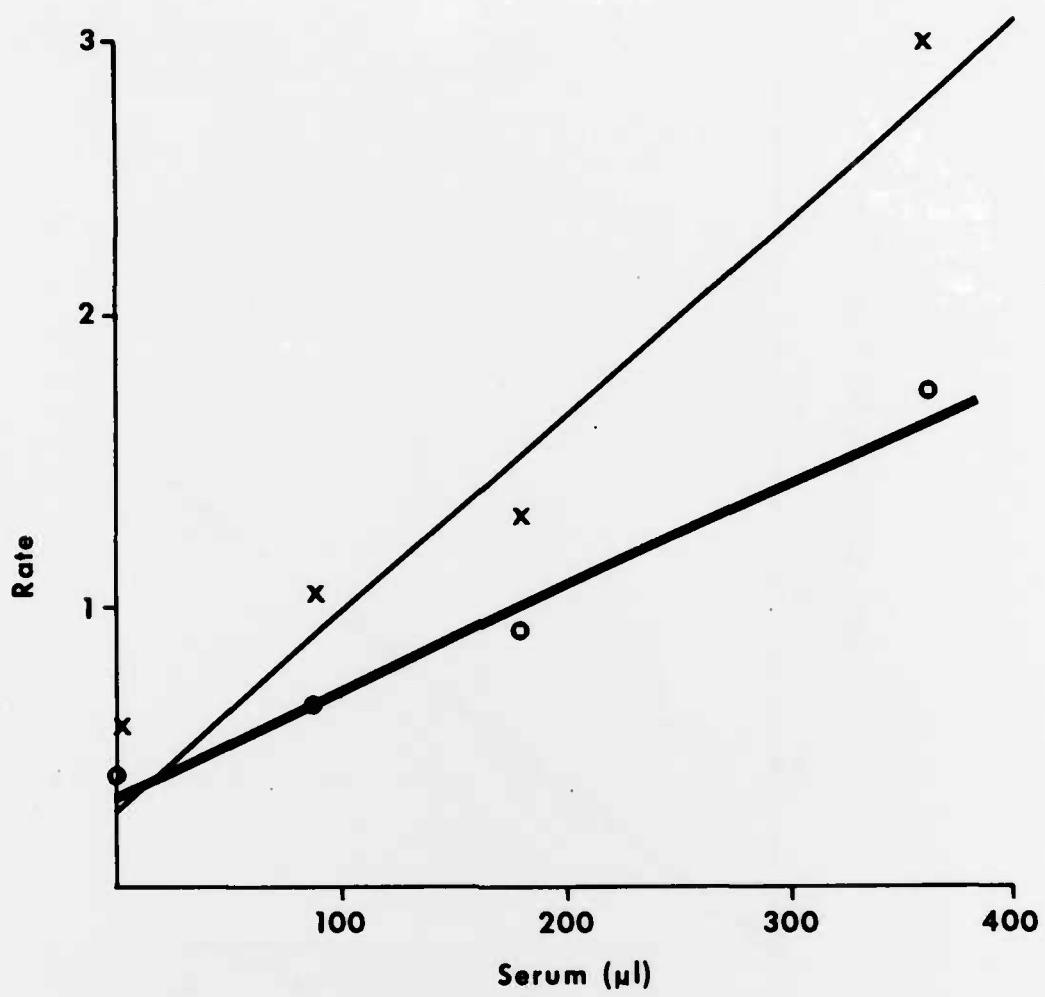


Figure 3. Dependence of rate of hydrolysis on pig serum concentration in glycine buffer pH 10.7. The paraoxon concentration was $1.23 \times 10^{-3} M$. The rate is given in moles per liter per minute.

x initial rate

o rate for the time interval of 20 min and 5 min

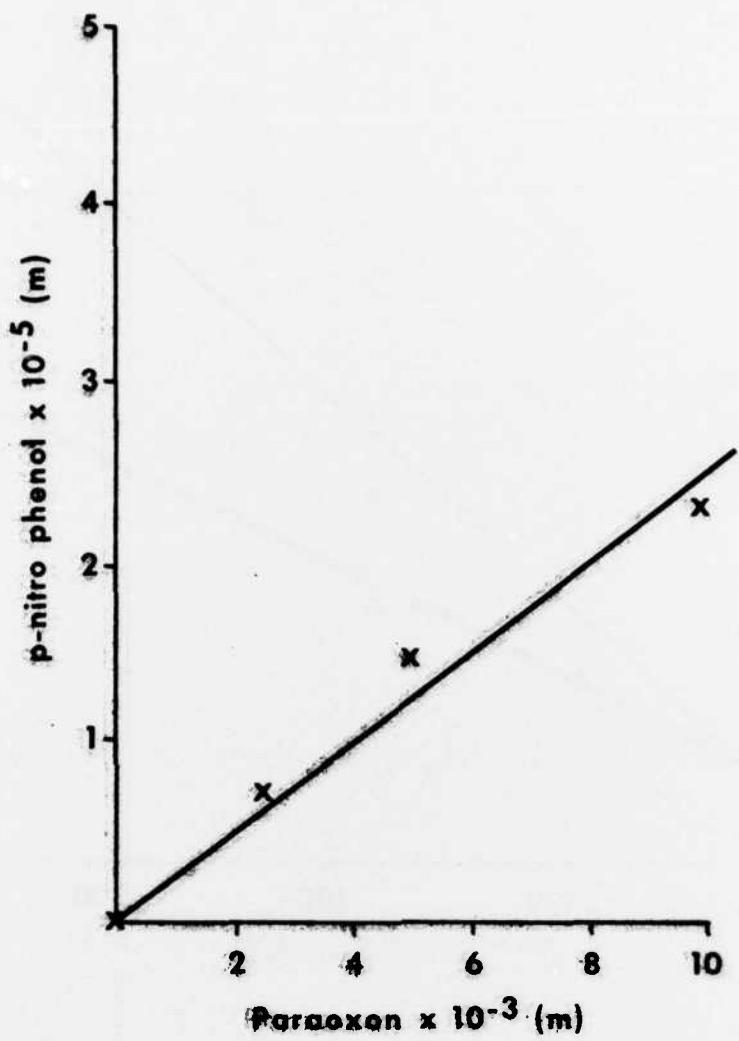


Figure 4. Hydrolysis of paraoxon with pig serum (3.4 mg/ml) in TRIS buffer pH 7.6.
4a. Initial liberation of p-nitro-phenol (*M*) for paraoxon concentrations (*M*).

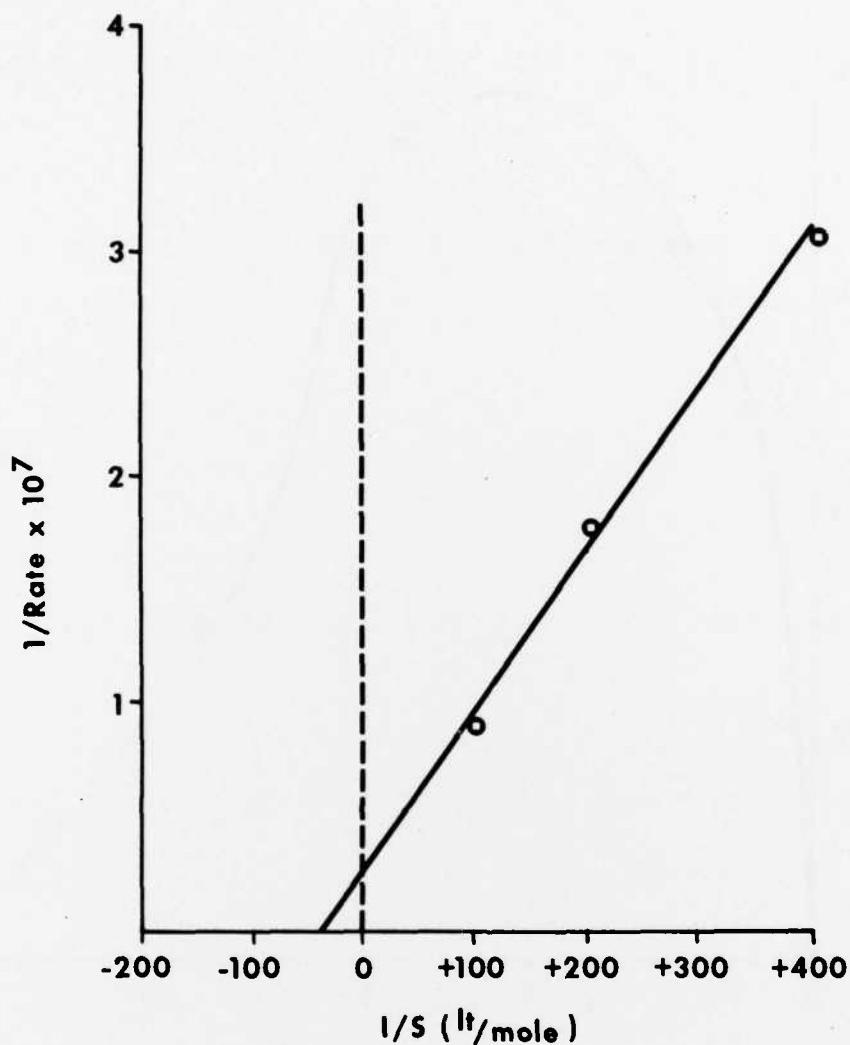


Figure 4. Hydrolysis of paraoxon with pig serum (3.4 mg/ml) in TRIS buffer pH 7.6. (concluded)
 4b. Lineweaver-Burk plot. The reciprocal rate ($1/\text{rate}$) is given in liters \times minutes per mole and the reciprocal concentration ($1/S$) is given in liters per mole.

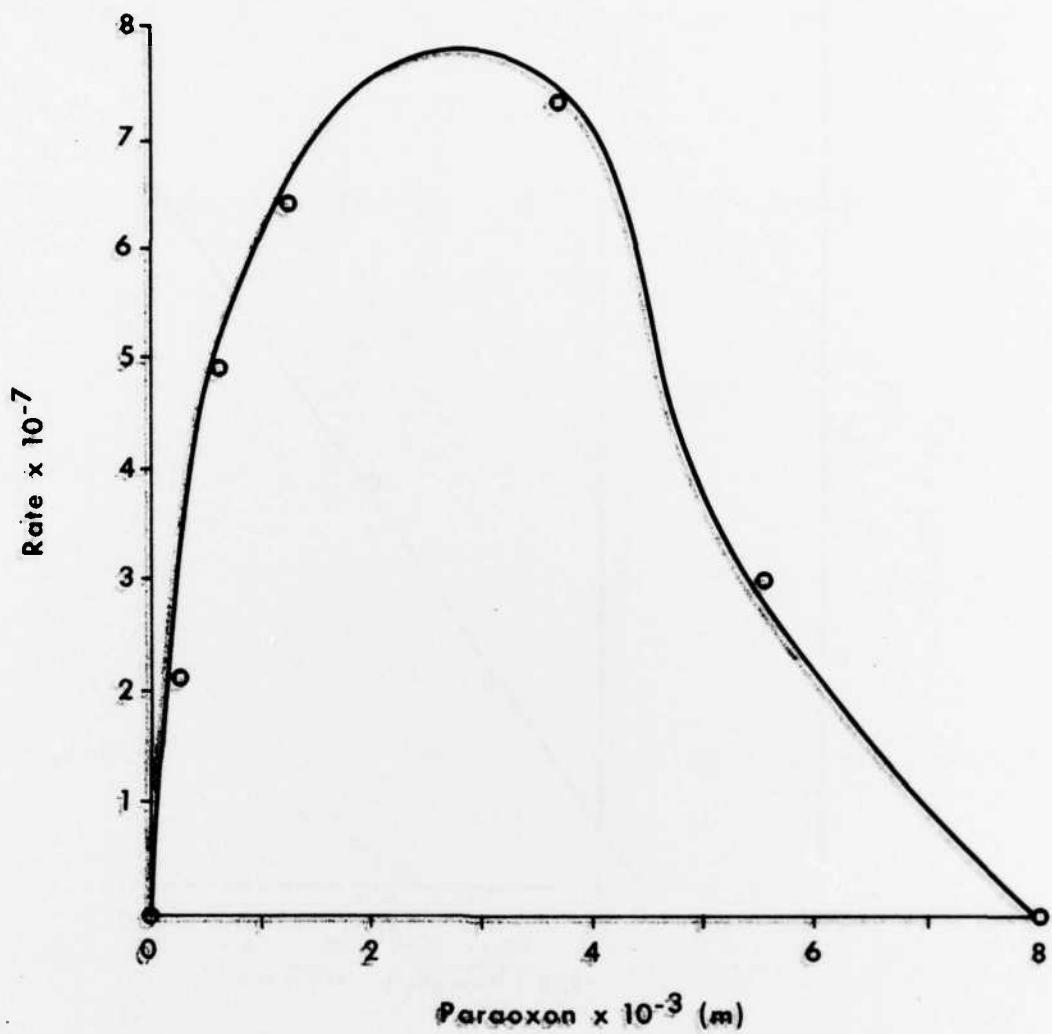


Figure 5. Influence of paraoxon concentration (M) on rate of hydrolysis (moles per liter per minute) in pig serum and glycine buffer (pH 10.5).

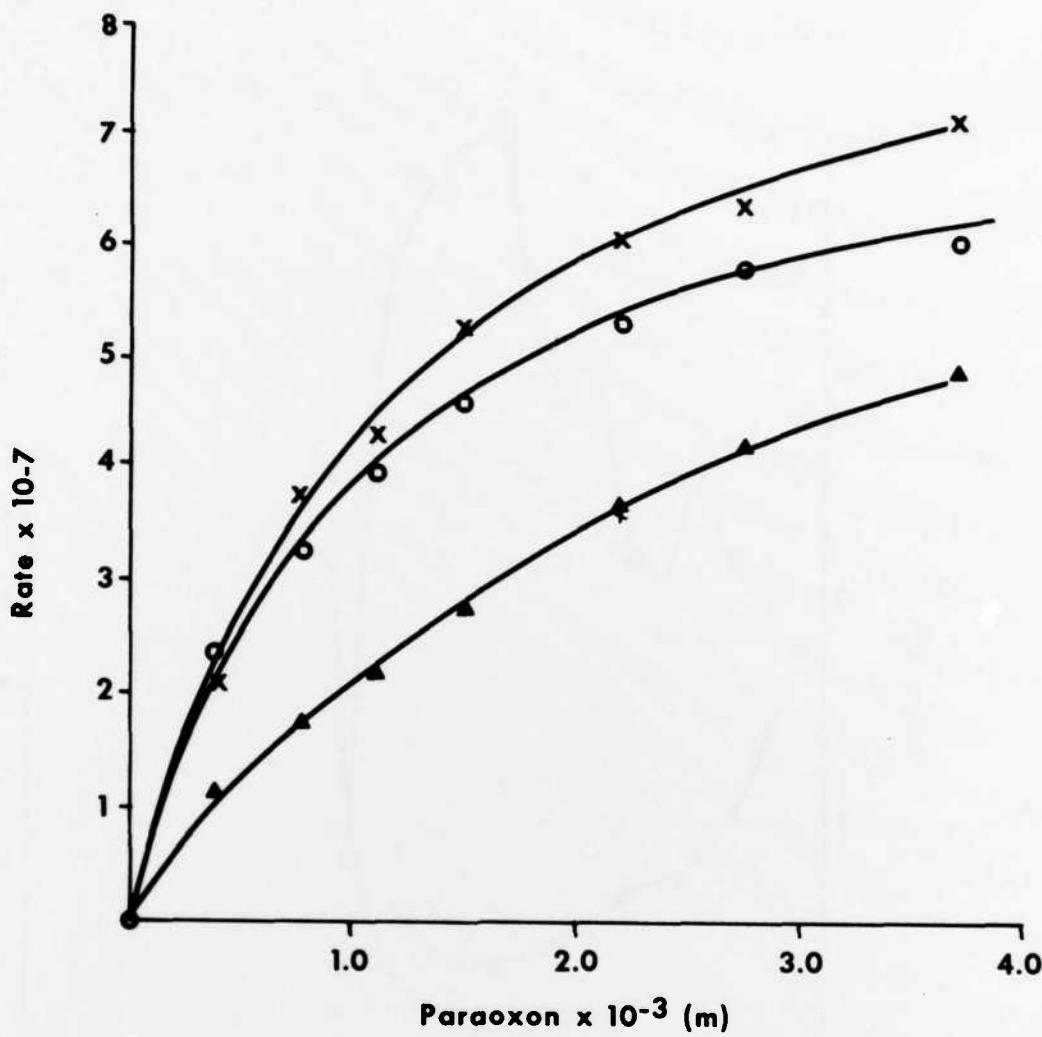


Figure 6. Influence of paraoxon concentration (M) on the rate of hydrolysis (moles per liter per minute) in pig serum. The symbol \circ indicates initial rate, \times is the rate for the time interval between 5.3 min and 0.3 min and \blacktriangle is the rate for the time interval between 20 min and 5 min. Measurements were made in glycine buffer (pH 10.5).

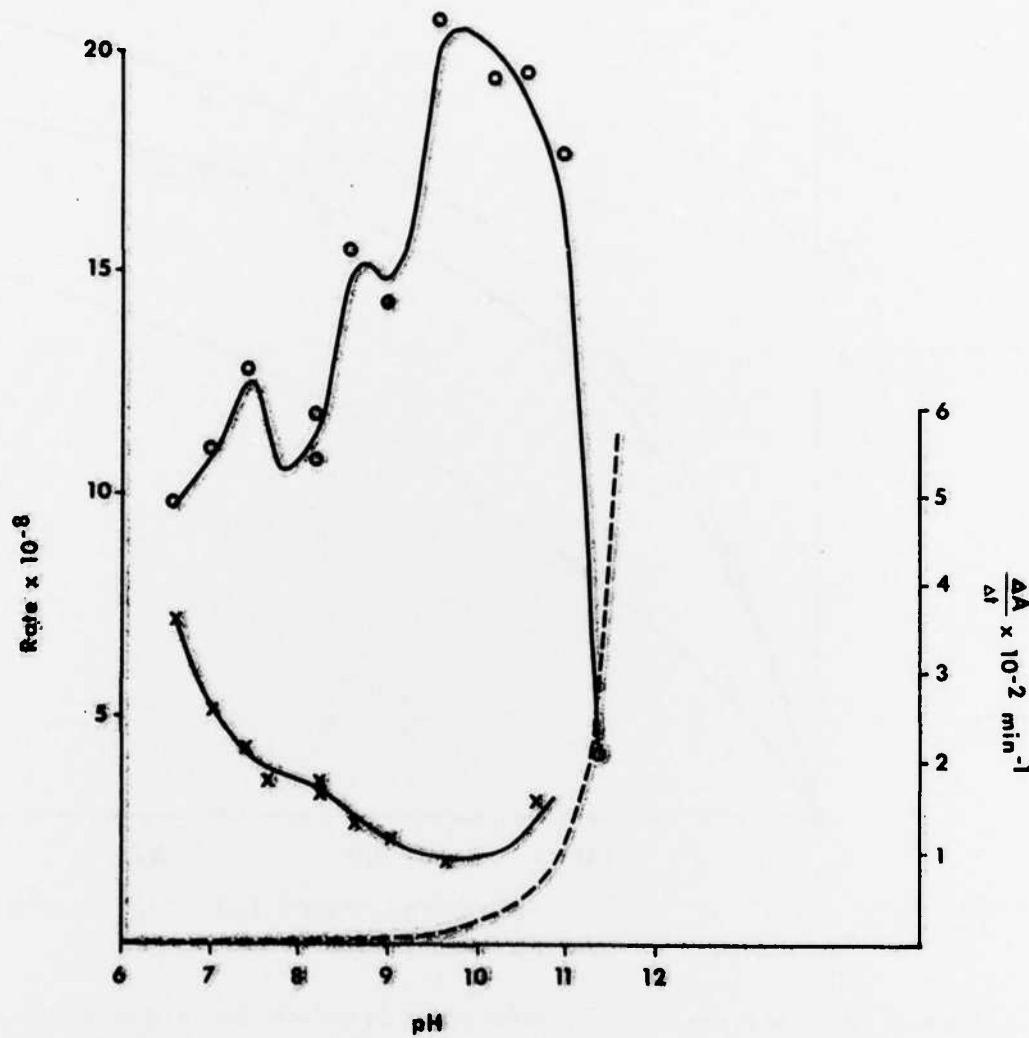


Figure 7. pH profile for the hydrolysis of pig serum (x) and human serum (o). Rate data are corrected for nonezymatic hydrolysis and are given in moles per liter per minute. The dashed curve (—) shows the liberation of p-nitro phenol (absorbance units per minute) of the buffer and paraoxon.

TABLES

Table 1. Hydrolysis of paraoxon in human serum.

Table 2. Influence of buffer ions on the hydrolysis of paraoxon in human serum.

Table 3. Michaelis-Menten (V_{max} and K_m) data for the hydrolysis of paraoxon by pig serum.

Table 4. Species comparison of hydrolysis of paraoxon in serum at two different pH's.

APPENDIX B

Table 1. Hydrolysis of paraoxon in human serum.

Initial rate with 3.7×10^{-4} M paraoxon in glycine buffer pH
10.6

Volunteer Serum	Rate* X 10^{-8})	
	180 ul serum	60 ul serum
Sc	1.9	2.4
Ka	5.4	4.7
Ru	7.6	6.4
Bl	5.3	5.2
Re	6.8	6.6

* The rate is given in moles per liter per minute per microliter serum.

Data are given for 180 microliter serum and 60 microliter serum.

Table 2. Influence of buffer ions on the hydrolysis of paraoxon in human serum.

Volunteer Serum	<u>Absolute Rate</u> [*]		<u>Relative Rate</u>		
	0.1 M Phosphate	0.1 M Phosphate	0.1 M Diethyl Malonate	0.1 M TRIS	0.01 M TRIS
B	6.4	1.0	0.47	0.65	0.99
J	1.6	1.0	0.47	0.91	1.52
S	0.8	1.0	0.64	0.81	1.37

* Paraoxon concentration 1.48×10^{-3} M.

The absolute rate is given in moles per liter per minute $\times 10^{-7}$.

Table 3. Michaelis Menten (V_{max} and K_m) data for the hydrolysis of paraoxon by pig serum.

time interval (min)	V_{max}	K_m
Initial	8.2×10^{-7}	5.7×10^{-4}
$t_{5.3} - t_{0.3}$	6.6×10^{-7}	3.5×10^{-4}
$t_{20} - t_5$	5.6×10^{-7}	4.0×10^{-4}

V_{max} data are given in moles per liter per minute and a pig serum concentration of 3.4 mg/ml.

K_m values molar

Table 4. Species comparison of hydrolysis of paraoxon* in serum at two different pH's.

Species	Rate of Hydrolysis X 10^{-8} ⁺			
	TRIS Buffer pH 7.5		Glycine Buffer [#] pH 10.5	
Fig	1.48	\pm 0.2 (4)*	4.71	\pm 2.10 (12)
Human Volunteers §	8.93	(4)	26.86	(1)
S	7.40	(1)		
J	20.13	(1)	24.95	
B	43.70	(1)		
mean #	20.0	(4)	25.9	(2)
Rat	---		4.01	\pm 2.21 (7)

* All determinations were made at a paraoxon concentration of 1.2×10^{-3} M.

+ Rate data are given in moles per liter per minute per mg serum.

Values in parenthesis indicates number of samples.

§ In view of the demonstration of a bimodal distribution (5) a standard deviation has not been calculated.

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